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Preparation and Properties of Hydrophilic Polypeptide Membranes as Biodegradable Materials

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Three-component random copolypeptides consisting of N-hydroxyalkyl-L-glutamine, L-glutamic acid, and L-lysine were prepared by carrying out aminoalcoholysis reaction with aminoalcohols, such as 2-amino-1-ethanol(E) and 4-amino-1-butanol(B), followed by crosslinking reaction with 1,8-octamethylenediamine (OMDA) on starting polymer membranes consisting of γ -methyl-L-glutamate, L-glutamic acid, and L-lysine. The effective crosslink density was shown to be proportional to the content of the crosslinker (OMDA) in the reaction mixture. The tensile properties of these hydrophilic membranes were highly dependent on the degree of swelling in the pseudo-extracellular fluid (PECF), hydrophobicity of the side chains, and the effective charge density of membranes, and their behavior was typical of an elastomer. A higher rate of water permeability was obtained with charged membranes than non-charged or compensated charged membranes with the same order of the degree of swelling in PECF. Biodegradation of the samples *in vitro* by actinase and papain indicated that the degradation could be regarded as a bulk rather than a surface phenomenon. The rate of degradation was also highly dependent on the degree of swelling of membranes, as well as on the hydrophobicity and effective charge density of side chains of the samples.

KEY WORDS: Charged Copolypeptide/ Water Permeability/ Enzyme/ Hydrophilic Polypeptide/ Membrane/ Biodegradable Materials/

INTRODUCTION

Poly(α -amino acid)s and their copolymers may be useful for biodegradable medical applications such as a; temporary artificial skin substitutes in burn therapy, b; temporary barriers to prevent adhesion between natural tissues planes damaged either by accident or surgery, and between the pericardium and heart wall during open-heart surgery, c; polymer carriers for conjugates coupled to proteins for therapeutic use, and d; in drug delivery systems.¹⁾ On the other hand, proteins contain both anionic and cationic groups in their molecules. Thus, it is interesting to investigate chain conformations as well as membrane properties of copolypeptides carrying both negative and positive charges in the side chains of the same molecules.

In this paper, we prepared three-component random copolypeptides (MBK) consisting of L-glutamic acid (B), L-lysine (K), and N-hydroxyalkyl-L-glutamine (M), as well as two-component random copolypeptides such as copoly (N-hydroxyalkyl-L-glutamine/L-glutamic acid)(MB) and copoly (N-hydroxyalkyl-L-glutamine/L-

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lysine)(MK), and homopolymers such as poly (L-glutamic acid), poly (L-lysine), poly (N-hydroxyethyl-L-glutamine) and poly (N-hydroxybutyl-L-glutamine), and investigated the relation between their bulk structures and membrane properties such as the degree of swelling in the pseudo-extracellular fluid (PECF), tensile properties in PECF, water vapor permeability, and enzymatic degradation behavior *in vitro* of the hydrophilic membranes in PECF, from application view points as biomedical materials.

EXPERIMENTAL

Materials

Synthesis of Copolypeptides. Three-component random copolypeptide, copoly(γ -methyl-L-glutamate/ γ -benzyl-L-glutamate/ ϵ -N-carbobenzoyloxy-L-lysine)(MBK), two-component random copolypeptides, copoly(γ -methyl-L-glutamate/ γ -benzyl-L-glutamate)(MB) and copoly (γ -methyl-L-glutamate/ ϵ -N-carbobenzoyloxy-L-lysine)(MK), and homopolypeptides, poly(γ -methyl-L-glutamate)(PMLG), poly(γ -benzyl-L-glutamate)(PBLG), and poly(ϵ -N-carbobenzoyloxy-L-lysine)(PCBL) were synthesized by the N-carboxyanhydride(NCA) method. The monomers, M-NCA, B-NCA, and K-NCA, were prepared according to the method reported in the previous paper,²⁾ and purified by recrystallization from an ethyl acetate solution with the addition of petroleum ether. Recrystallization was repeated more than three times. The polymerization was initiated with triethylamine (TEA) at an NCA-to-TEA molar ratio of 50. All starting polymers were purified and fractionated as described in the previous paper.³⁾ The results of all the polymerizations are summarized in Table I.

Table I. Copolymerization of random copolypeptides

Sample Code	NCA (mol-%)			$[\eta]$ (dl/g) (DCA, 25°C)	M_w
	M	B	K		
MBK-11	90	5	5	1.69	323,000
MBK-21	80	15	5	1.44	252,000
MBK-22	80	10	10	1.56	281,000
MBK-23	80	5	15	1.89	367,000
MBK-32	70	15	15	1.44	248,000
MB-1	90	10	0	2.01	375,000
MB-2	80	20	0	1.98	371,000
MK-1	90	0	10	1.55	276,000
MK-2	80	0	20	1.26	176,000
PMLG-1	100	0	0	0.98	163,000
PBLG-1	0	100	0	1.22	224,000
PCBL-1	0	0	100	1.88	312,000

Preparation of Hydrophilic Polymer Membranes. The debenzoylation of γ -BLG as well as the decarbobenzylation of ϵ -CBL residues in copolymers was performed by anhydrous HBr treatment according to the method of Idelson and Blout.⁴⁾

After a membrane of ca. 100 μ m in thickness cast from an appropriate mixed solvent was immersed in a mixture of 2-amino-1-ethanol (E) and the crosslinker 1,8-octamethylenediamine (OMDA), as well as in a mixture of 4-amino-1-butanol (B), n-butanol, and OMDA, at 60°C for 48 hr, the membrane was rinsed with pure water, ethyl ether, and stored in pure ethanol. Figure 1 shows a schematic diagram for the preparation of the hydrophilic membranes.

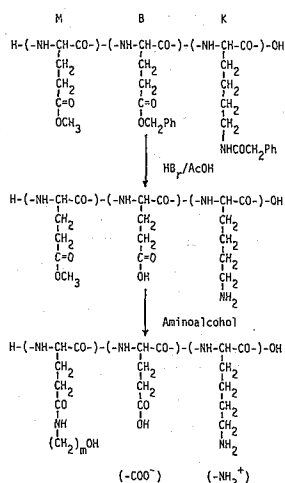


Fig. 1. Schematic diagram for preparation of hydrophilic membranes.

Measurements

Molecular Characterization of the Starting Copolymers. The molecular weights of the starting polymers were determined in N, N'-dimethylformamide (DMF) or in m-cresol at 25°C by sedimentation equilibrium using a MOM Type-3170-b ultracentrifuge equipped with a Rayleigh interference optical system and a 12 mm double sector cell.⁵⁾ The intrinsic viscosity was measured with dichloroacetic acid (DCA) solution, using an Ubbelohde type viscometer at 25°C. These data are also listed in Table I.

The chain conformation of the hydrophilic polymers in PECF was examined by optical rotatory dispersion (ORD) measurements with water-soluble samples prepared without crosslinker OMDA. The Moffitt-Yang parameter b_0 was evaluated from the ORD data obtained with a Yanagimoto OR-100 Type spectrophotometer, using a tungsten lamp at 37°C. Table II summarized the experimental data of b_0 for MBK (E), MB (E), MK (E), MBK (B), MB (B), MK (B), PHEG, PHBG, poly-L-glutamic acid, and poly-L-lysine, in PECF at pH 7.4 and 37°C.

Table II. Moffitt parameter b_0 for samples in PECF (pH 7.4, 37°C)

Sample Code	Starting Polymer	b_0
MBK (E)-110	MBK-11	-30
MBK (E)-220	MBK-22	-45
MBK (E)-320	MBK-32	-45
MB (E)-20	MB-2	-30
MK (E)-20	MK-2	-15
PHEG-10	PMLG-1	-30
MBK (B)-110	MBK-11	-270
MBK (B)-220	MBK-22	-260
MB (B)-20	MB-2	-150
MK (B)-20	MK-2	-180
PHBG-1	PMLG-1	-290
PLGlu-10	PBLG-1	60
PLLys-10	PCBL-1	40

Table III. Preparative data of hydrophilic membranes

Sample Code	Starting Polymer	OMDA (mol-%)	Q_w (%)
MBK (E)-221	MBK-22	1.0	1350
MBK (E)-222	MBK-22	2.0	960
MBK (E)-223	MBK-22	3.0	750
MBK (E)-223	MBK-22	4.0	630
MBK (E)-224	MBK-22	4.0	630
MB (E)-22	MB-2	2.0	1200
MB (E)-23	MB-2	3.0	980
MB (E)-24	MB-2	4.0	800
MK (E)-22	MK-2	2.0	1250
MK (E)-23	MK-2	3.0	1010
PHEG-11	PMLG-1	1.0	1150
PHEG-12	PMLG-1	2.0	790
PHEG-13	PMLG-1	3.0	560
PHEG-14	PMLG-1	4.0	480
MBK (B)-221	MBK-22	1.0	920
MBK (B)-222	MBK-22	2.0	630
MBK (B)-223	MBK-22	3.0	500
MB (B)-21	MB-2	1.0	1210
MB (B)-22	MB-2	2.0	840
MB (B)-23	MB-2	3.0	620
PHBG-11	PMLG-1	1.0	850
PHBG-12	PMLG-1	2.0	550
PHBG-13	PMLG-1	3.0	460

Physical Properties of Hydrophilic Membranes. The degree of swelling Q_w in PECF was determined by equilibrating the membrane in PECF at 37°C. The membrane taken out of PECF was blotted to remove surface PECF, and weighed until a constant weight was achieved. The membrane was then dried in a vacuum oven. The Q_w was defined as the ratio of the weight of PECF in swollen sample to the weight of the dried crosslinked hydrophilic membrane. Table III summarized experimental data of Q_w for membranes with the different molar ratio of OMDA in aminoalcohol solution.

The tensile properties of hydrophilic membranes were measured in PECF at 25°C by a Tensilon UTM-II-20 (Toyo-Boldwin Co.) using the standard techniques. All the samples were tested at an elongation rate of 40% per minute.

Water vapor permeation through the membranes was measured with a cylindrical glass cell⁽⁶⁾ at 37°C. The exposed membrane area was 12.57 cm².

Biodegradation of Hydrophilic Membranes in vitro. Enzymatic degradation studies *in vitro* were carried out by using papain and actinase. These enzymes were purchased from Nakarai Chem. Co. Enzyme solution were prepared by standard techniques⁽⁷⁾ at 37°C. A series of the crosslinked hydrophilic membranes were exposed to PECF solution at pH 7.4 with appropriate activators at 37°C. Polymer membranes were removed from the enzyme solution at appropriate time intervals, weighed, and then vacuum dried at 60°C to constant weights.

RESULTS AND DISCUSSION

Molecular Conformations of Copolymers in PECF

In Table II, it is shown that PHEG homopolymer exists in random coil conformation at pH 7.4 and 37°C in PECF, while poly-L-glutamic acid and poly-L-lysine exist in charged coil conformation.⁽⁸⁾ On the other hand, PHBG exists in an interrupted α -helix conformation at the same experimental condition.

The equimolar-charged (molar ratio of B to K is unity) hydrophilic membranes, MBK (B)-110 and MBK (B)-220, exist in the same content of the interrupted α -helix conformation as PHBG, indicating that the interaction between B and K residues leads to helix stabilization.⁽⁹⁾ While, the helix content of MB (B)-20 and MK (B)-20 was, respectively, less than that of MBK (B)-220 according to the charge effect on the helix destabilization.

Degree of Swelling of Hydrophilic Membranes in PECF

The degree of swelling in a solvent is determined by the free energy of mixing between the solvent and polymer and the elastic free energy consequential to the expansion of the network structure in the solvent.

The precise crosslink density has not been determined because of the uncertainty in the relative reactivity of aminoalcohol to OMDA, and also because of the difficulty to estimate the fraction of reacted diamine molecules which form effective crosslinks. However, here the effect of crosslinker (OMDA) concentration in the reaction mixture

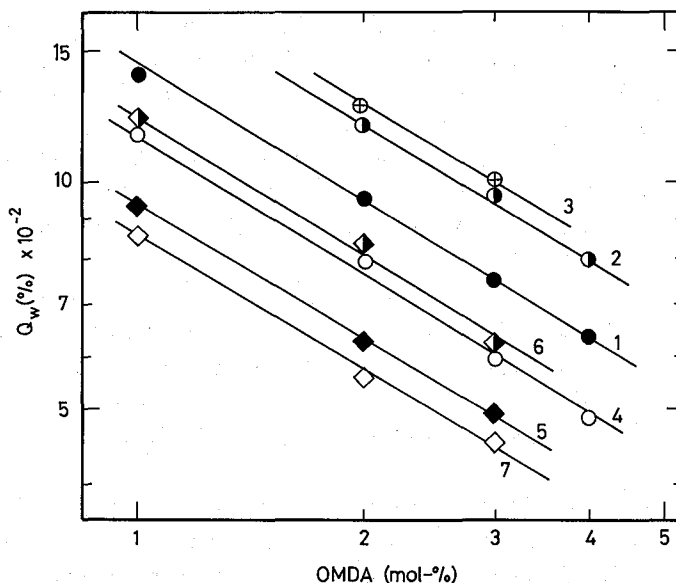


Fig. 2. The degree of swelling Q_w (%) of hydrophilic membranes in PECF against the molar percent of OMDA in double logarithmic scale: (1) MBK(E) (\circ), (2) MB(E) (\bullet), (3) MK(E) (\oplus), (4) PHEG(\circ), (5) MBK(B) (\blacklozenge), (6) MB(B) (\diamond), and (7) PHBG(\diamond).

on the degree of swelling Q_w of the crosslinked membranes in PECF is shown in Figure 2. The degree of swelling Q_w in PECF decreases with increasing OMDA molar concentration in the reaction solution.

If the degree of swelling Q_w is quite large, the following equation holds according to rubber elasticity theory¹⁰⁾

$$Q_w^{5/3} = (\bar{v}M_c)(1 - 2M_c/M)^{-1}(1/2 - \chi_1)/V_1 \quad (1)$$

where M_c is the molecular weight per crosslinked unit, M the primary molecular weight, \bar{v} the specific volume of polymer, V_1 the molar volume of solvent, and χ_1 the interaction parameter. The factor $(1 - 2M_c/M)$ expresses the correction for network imperfections resulting from chain ends. For a quite high molecular weight polymer chain, it reduces to unity. As the effective crosslink density f_c is proportional to the value of M_c/M where M_o is the molecular weight of the repeating unit (monomeric unit), equation (1) may be simplified as

$$Q_w^{5/3} = K(\bar{v}M_o)(1/2 - \chi_1)/V_1 f_c \quad (2)$$

where K is a constant. The slope of log-log plots in Figure 2 leads to a value of $-3/5$ as predicted by equation (2). This means that the effective crosslink density f_c is proportional to the crosslinker concentration (mol %) in the reaction solution. The Q_w values obtained with the charged membranes MB (E) and MK (E) were higher than those obtained with the compensated charged membranes, MBK (E), as well as the non-charged membranes, PHEG, of the same order of OMDA molar concentration,

indicating that Q_w value depends on the charge density in membranes.

Tensile Properties of Hydrophilic Membranes in PECF

The tensile properties of hydrophilic membranes are highly dependent on the degree of swelling in PECF. Further, elastomeric membranes are highly suited to biomedical applications, such as membranes for artificial organs, reconstructive prosthesis, and cosmesis.

Figure 3 illustrated the stress-strain curves of hydrophilic membranes in PECF at 25°C. While MB (E)-24 and PHEG membranes gave lower strength, MBK (E)-222 attained higher strength with moderate modulus. Table IV lists the experimental findings of Young's modulus E at an elongation of 1%, the tensile strength δ_B and elongation ϵ_B at the breaking point together with Q_w values for membranes in PECF. Tensile strength of MBK (B)-221 membrane is higher than that of PHBG-11, even though the Q_w value of MBK (B)-221 is higher than that of PHBG-11, showing that the charge interactions between cation and anion in the molecular chains affect the tensile strength of the membrane. Further, it is pointed out that the degree of swelling in PECF for membranes containing N-hydroxybutyl-L-glutamine (B) becomes much lower than those for membranes containing N-hydroxyethyl-L-glutamine (E) (see Figure 2) at the same order of OMDA molar concentration. The hydrophobic nature of glutamine side chain increases from E to B. Lotan, et al.¹¹⁾ have shown that the helix content in

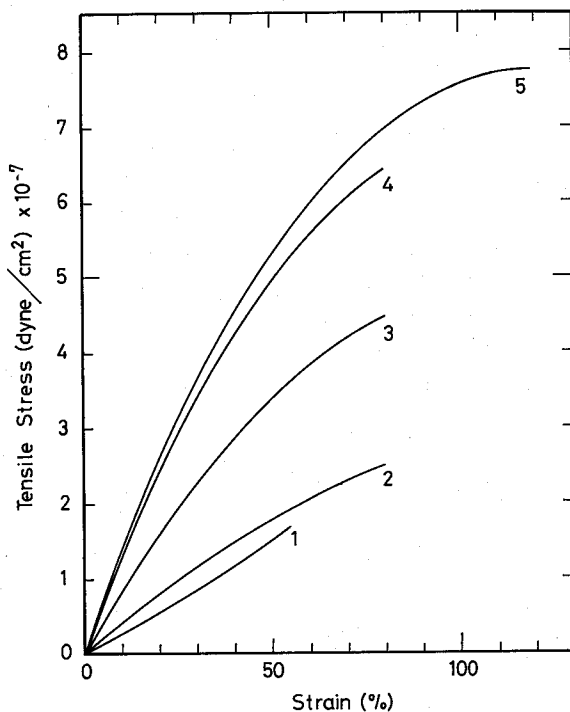


Fig. 3. Stress-strain behaviors of membranes in PECF at 25°C: (1) MB(E)-24, (2) PHEG-12, (3) MBK(E)-222, (4) PHBG-12, and (5) MBK(B)-221.

Table IV. Mechanical properties of membranes in PECF at 25°C

Sample Code	Q_w (%)	E (dyne/cm ²)	δ_B (dyne/cm ²)	ϵ_B (%)
MBK (E)-222	960	8.4×10^7	4.5×10^7	80
MBK (E)-224	630	1.2×10^8	5.9×10^7	80
MB (E)-22	1200	1.8×10^7	1.0×10^7	40
MB (E)-24	800	2.5×10^7	1.7×10^7	55
MK (E)-23	1010	1.9×10^7	1.1×10^7	36
PHEG-11	1150	3.0×10^7	1.8×10^7	75
PHEG-12	800	4.3×10^7	2.5×10^7	80
MBK (B)-221	920	1.5×10^8	7.8×10^7	120
MBK (B)-222	630	2.1×10^8	9.8×10^7	95
MB (B)-21	1210	2.6×10^7	1.6×10^7	70
MB (B)-22	840	3.7×10^7	2.6×10^7	85
PHBG-11	850	8.0×10^7	4.1×10^7	90
PHBG-12	550	1.4×10^8	6.5×10^7	80

aqueous solution increases with increasing length of hydrocarbon of side chain, and PHBG exists in the interrupted α -helix conformation, while PHEG exists in random coil conformations. Thus, the Q_w value may be affected by molecular conformation.

Furthermore, it is pointed out from Figure 3 as well as from Table IV that the hydrophobic nature of the glutamine side chain affected the mechanical properties of membranes.

Water Vapor Permeability of Membranes

A large variety of synthetic polymer membranes has been investigated in the treatment of burns.¹²⁻¹⁴⁾ Among them, for example, the formulation of a crosslinked polymer in the form of hydrogel appears to have added capability for encouraging cellular migration into the graft and vascularization.¹⁵⁾ In designing an effective wound closure or an artificial skin inner-layer substrate, at least two functions of skin are urgently essential for survival. The first is the ability of skin to keep most bacteria out. The second is its ability to control water passage moderately from tissue and organ. Thus, it is important to know the value of the rate of water vapor permeability V_f through the membrane. If the V_f value is excessively low, water accumulates at the interface between the wound-bed and impermeable graft, and edema results. The graft/wound-bed interfacial contact is thereby undermined. Accordingly, to maintain the ability to wet the wound-bed and thereby maintain an air-free interface, an inner skin substitute membrane should have a higher V_f value than that of the human physiological level of about 500 g/m² day.

Figure 4 illustrates the relation between the rate of water vapor permeability V_f of PECF and the degree of swelling Q_w for membranes at 37°C. It may be also shown that the V_f value is highly dependent on the Q_w value in PECF. Further, it may be judged in Figure 4 that the V_f value of the charged membranes, MB (E) and MK (B), is

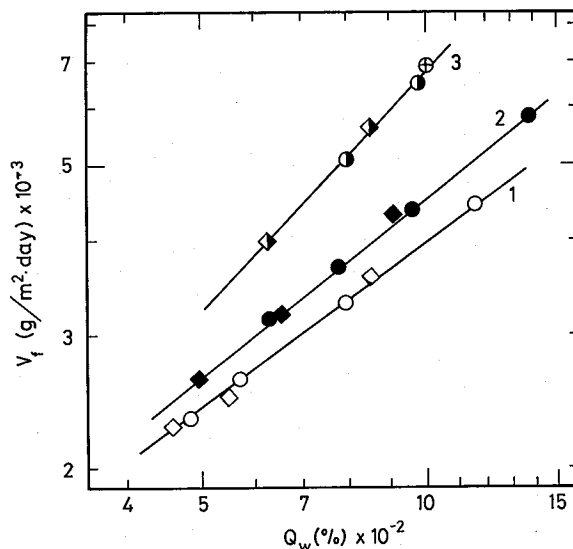


Fig. 4. Rate of water vapor permeability V_f (g/m^2 day) against the degree of swelling Q_w (%) in double logarithmic scale: (1) PHEG(○) and PHBG(◇), (2) MBK(E) (●) and MBK(B) (◆), and (3) MB(E) (●), MB(B) (◆), and MK(E) (⊗).

higher than those of the compensated charged or non-charged membranes, MBK (E), MBK (B), PHEG, or PHBG, at the same value of Q_w , indicating that the charge density in membranes affects the state of water in membranes.

Biodegradation of Membranes in vitro

Numerous proteases may be present at a wound site.¹⁶⁾ These proteases are divided into some classes depending on the structure of active sites. Enzymes of inflammatory response that are likely to degrade poly(α -amino acid)s include the endopeptidase Cathepsin B and the exopeptidases Carboxypeptidase and Leucine amino peptidase.¹⁷⁾ In the present investigation, a plant thiol endopeptidase papain, as a commercially available analog of Cathepsin B, and actinase, as a commercially available analog of an exopeptidase released during the acute and chronic stages of the inflammatory response, were used. Although papain is a general plant thiol endopeptidase, the mechanism of the degradation of peptide bonds with papain was reported to be similar with that with Cathepsin B.¹⁸⁾ Actinase obtained from *Streptomyces Griseus* has been separated into eight proteases¹⁹⁾ of which four are serine endopeptidases.²⁰⁾ These endopeptidases are not expected to hydrolyze N-hydroxyalkyl-L-glutamine series. The remaining four proteases are exopeptidases; two are aminopeptidases²¹⁾ and two are carboxypeptidases.²²⁾ The actinase activity observed in the crosslinked membranes in this study almost certainly comes from these exopeptidases.

Pre-weighed MBK (E)-224 membranes were exposed to papain or actinase and the results are illustrated in Figure 5. The dry weights of these membranes began to decrease immediately and the weight loss continued steadily thereafter for actinase

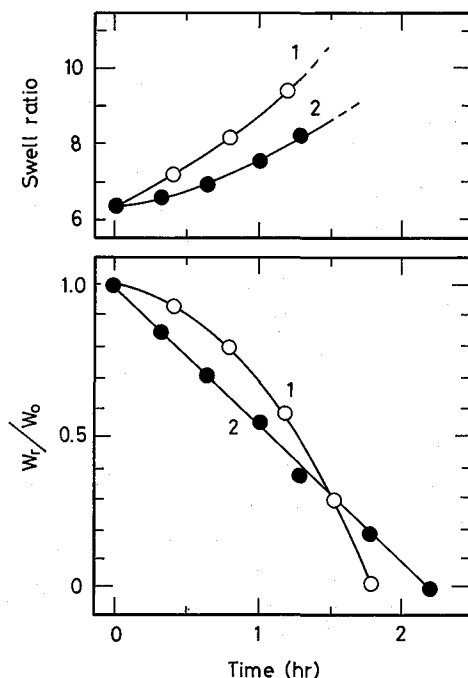


Fig. 5. Dry weight ratio (W_t/W_0), and swelling ratio in PECF for MBK (E)-224 membrane against enzymatic digestion time (hr): (1) papain (○) (0.15 mg/ml) and (2) actinase (●) (0.4 mg/ml) at 37°C and pH 7.4.

system. Simultaneously, the swelling ratio increased steadily. On the other hand, the MBK (E)-224 membrane that was exposed to papain showed a lag time with respect to weight loss so that when the experiment was half over, i.e., at half the time needed to dissolve the specimen, about 75% of the dry weight remained. The increase in swelling ratio for papain system was always higher order than that for actinase system. Judging from the immediate increase in the swelling ratio, the degradation of the MBK (E)-224 membrane should be a bulk rather than a surface phenomenon. Qualitative differences in the action of actinase and papain are clearly shown in Figure 5. The immediate and steady weight loss observed with actinase system is consistent with the release of monomers from the chain ends by exopeptidases. On the other hand, an endopeptidase, such as papain, must make two incisions in a chain segment if a soluble fragment is produced, but a single cleavage will decrease the effective crosslink density, resulting in increased swelling ratio of membranes. Thus, the initial effect of papain is therefore to decrease the effective crosslink density without producing soluble materials.

Figures 6 and 7 summarize the rate of papain and actinase digestion, $V(1/2)(h^{-1})$, respectively, as a function of the degree of swelling Q_w in PECF for hydrophilic polymer membranes. $V(1/2)$ is defined as the reciprocal of the time required for the sample weight to be reduced to one-half its initial value. It is clearly shown that the order of degradation rates among the hydrophilic membranes is as follows: MBK(B) >

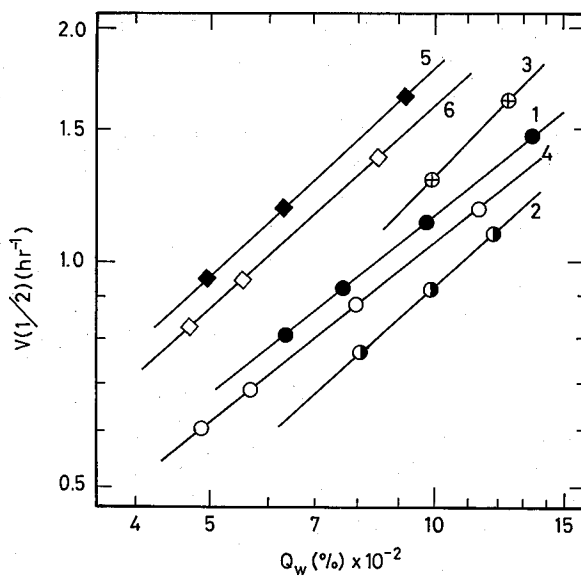


Fig. 6. Rate of papain digestion $V(1/2)$ (h^{-1}) against degree of swelling Q_w (%) in double logarithmic scale: (1) MBK(E) (\bullet), (2) MB(E) (\bullet), (3) MK(E) (\otimes), (4) PHEG(\circ), (5) MBK(B) (\blacklozenge), and (6) PHBG(\diamond).

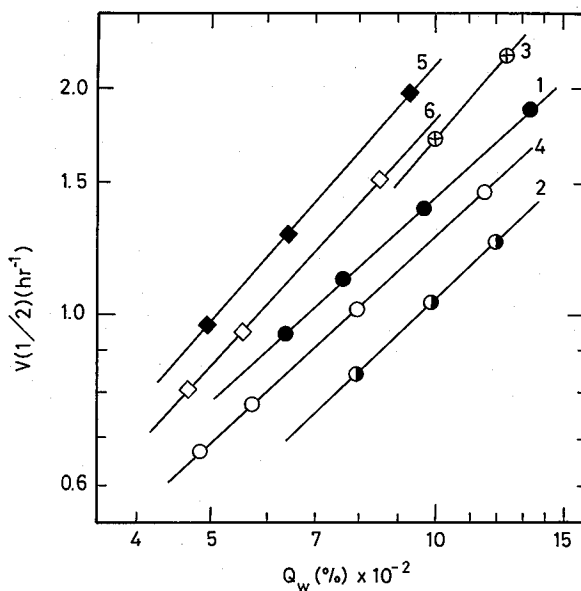


Fig. 7. Rate of actinase digestion $V(1/2)$ (h^{-1}) against degree of swelling Q_w (%) in double logarithmic scale: (1) MBK(E) (\bullet), (2) MB(E) (\bullet), (3) MK(E) (\otimes), (4) PHEG(\circ), (5) MBK(B) (\blacklozenge), and (6) PHBG (\diamond).

PHBG > MK(E) > PHEG > MBK(E) > MB(E) at the same order of Q_w for each membrane.

In conclusion, the effective crosslink density was shown to be proportional to the

content of the crosslinker (OMDA) in the reaction mixture. Q_w values were highly dependent on the charge density in membranes. The tensile properties of these hydrophilic membranes depended on the Q_w in PECF, the charge interactions between cations and anions in the molecular chain, and the molecular conformation of chains in membranes, and their behavior was typical of an elastomer. Biodegradation of these hydrophilic membranes by actinase and papain *in vitro* indicated that the degradation could be regarded as a bulk rather than a surface phenomenon. The rate of degradation of sample was also highly dependent on Q_w of membranes, as well as on the charge density of the sample.

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